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Immunomagnetic separation of circulating tumor cells with microfluidic chips and their clinical applications

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ABSTRACT

Circulating tumor cells (CTCs) are tumor cells detached from the original lesion and getting into the blood and lymphatic circulation systems. They potentially establish new tumors in remote areas, namely, metastasis. Isolation of CTCs and following biological molecular analysis facilitate investigating cancer and coming out treatment. Since CTCs carry important information on the primary tumor, they are vital in exploring the mechanism of cancer, metastasis, and diagnosis. However, CTCs are very difficult to separate due to their extreme heterogeneity and rarity in blood. Recently, advanced technologies, such as nanosurfaces, quantum dots, and Raman spectroscopy, have been integrated with microfluidic chips. These achievements enable the next generation isolation technologies and subsequent biological analysis of CTCs. In this review, we summarize CTCs' separation with microfluidic chips based on the principle of immunomagnetic isolation of CTCs. Fundamental insights, clinical applications, and potential future directions are discussed.

I. INTRODUCTION

Circulating tumor cells (CTCs),[1] discovered by an Austrian physicist, Thomas Ashworth, in 1869,[2] are cancer cells shed from the primary or secondary lesion. They enter into the blood and lymphatic circulation systems, spreading and forming distant tissues.[3–5] The behavior of those CTCs might finally lead to cancer metastasis, responsible for almost 90% death of cancer patients.[6–8] Thus, the detection of CTCs in the blood sample indicates cancer and possible metastasis. Also, since CTCs carrying significant molecular information from the primary tumor,[9] detecting and characterizing CTCs would contribute to the understanding of cancer and its metastasis mechanism. Further, the number of CTCs is related to the overall survival of patients and the severity of cancer,[10–14] as less number indicates disease improvement or effective treatment. Thus, the precise enumeration of CTCs has endowed more significant values in prognosis and treatment procedures. It could be seen that CTCs have prognostic relevance and clinical meaning.[15–18]

As seen in Fig. 1, microfluidic chips are non-invasive or conservative "liquid biopsy" detectors.[19–21] This technology would minimize the pain in cancer detection. However, CTCs are extremely rare and heterogeneous, with approximately 1–10 CTCs amid millions of white blood cells (WBCs) and billions of red blood cells (RBCs) in 1 ml whole blood.[22,23]

Until now, Cellsearch™ (developed by Veridex Raritan, NJ, USA) is the only device approved by the US Food and Drug Administration (FDA) for successful clinical CTC enumeration system in breast, prostate, and colorectal cancers.[24–27] As reported, the efficiency of this system was approximately 80%.[28] In this device, immunomagnetic technology is through ferrofluidic nanoparticles labeled with antibodies targeting epithelial cell adhesion. CTCs are identified through fluorescent staining of cytokeratins proteins of keratin-containing intermediate filaments in the intracytoplasmic cytoskeleton of epithelial tissue. Leukocytes are excluded by CD45 (Leucocyte Common Antigen) antibody staining. To our knowledge, the main limitation of CellSearch™ lies in the ineffectiveness of cells with low expression of epithelial markers. This ineffectiveness affects cell viability and further downstream characterization. Also, some

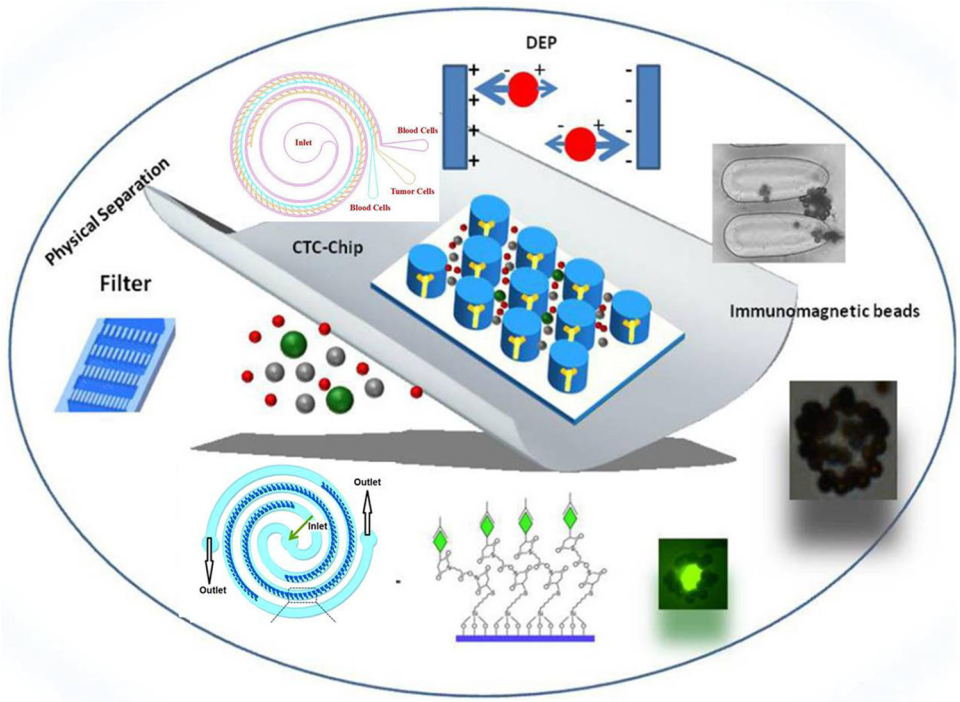

FIG. 1. Schematic of all kinds of microfluidic chips for isolating circulating tumor cells.

leukocytes of epithelial expression would cause false-positive results. Besides, it is still semi-automatic with low efficiency and high cost.

Besides the commercialized products, more advanced progress has been made toward capturing and characterizing CTCs with microfluidic chip-based technology. Microfluidic chips have unique advantages over conventional approaches. For example, in the separation of CTCs using density gradient centrifugation, the fluid media, Ficoll–Hypaque, is toxic to cells.[29] Flow cytometry always requires pre-enrichment. But the cell loss may occasionally happen in the pre-separation stage. On the other hand, microfluidic chips could integrate different stages on a small chip, which exhibits high potential applications in relevant technologies such as DNA sequencing,[30,31] protein detection,[32,33] cell manipulation, and molecular analysis.[34,35] Besides, materials for microfluidic chips, such as PDMS, are always biocompatible and low-cost. All these merits make microfluidic chips one of the best candidates for cell-scale CTCs sorting.

Until now, there have been few reviews about the immunomagnetic separation of CTCs with microfluidic chips and their clinical application. We analyze each typical microfluidic chips and its working principle as well as clinical assays. Some viewpoints described in the manuscript, such as reproducibility, clinical assay, advantages and disadvantages, and future prospective, are new. From those points of view, this review manuscript is unique. In this paper, we reviewed emerging immunomagnetic-based microfluidic chips designed for capturing and isolating CTCs. Prospective potential research interests of CTCs' capture based on this principle are presented and discussed.

II. CURRENT MICROFLUIDIC CHIP-BASED METHODOLOGIES FOR ISOLATION OF CTCs

A. Current technologies

To date, the CTC microfluidic chips can be classified into the following three types according to different working principles:

(1) Immunoaffinity-based, which depends on the specific binding of antigens of CTCs to antibodies modified on microchannels of the microfluidic chip.[36–54]
(2) Physical-based, which depends on the physical property differences, such as size/deformability discrepancy between CTCs and hematological cells.[55–73]
(3) Immunomagnetic-based, in which CTCs are bonded with immunomagnetic beads with size amplification and magnetization.[74–82]

Most technologies, such as dielectrophoresis-based,[83–86] acoustic-based, electrophoresis-based,[87,88] hydrodynamic and cross-flow filtration,[89,90] deterministic lateral displacement (DLD),[91,92] and inertial focusing systems,[93–95] including the viscoelasticity aided method,[96] fall into these three categories.

B. Separation evaluation

Parameters used to evaluate the efficiency of microfluidic chips are as follows:

$$\text{Recovery rate (\%)} = [(\text{captured tumor cells})/(\text{spiked tumor cells})] \times 100\%, \quad (1a)$$

$$\text{capture efficiency (\%)} = [(\text{captured CTCs})/(\text{captured} + \text{escaped CTCs})] \times 100\%, \quad (1b)$$

$$\text{capture purity (\%)} = [(\text{captured CTCs})/(\text{captured CTCs} + \text{captured WBCs})] \times 100\%, \quad (2)$$

$$\text{release efficiency (\%)} = [(\text{released CTCs})/(\text{captured CTCs})] \times 100\%, \quad (3)$$

$$\text{viability (\%)} = [(\text{viable CTCs})/(\text{viable CTCs} + \text{apoptosis CTCs})] \times 100\%. \quad (4)$$

The reason for formula (1b) seldom getting involved in capture efficiency (CE) calculation, but more precise is due to sedimentation. From rich experience, we could conclude that formula (1b) could better reflect the real value. As CTCs are usually heavier than blood constitutes with bigger size, CTCs tend to precipitate in the front part of the syringe.[97] In particular, in the case of slow flow, it would cause the total amount of CTCs captured and escaped from the outlet is less than the number of CTCs spiked in. The sedimentation phenomenon could be observed if tumor cells coated with dark immunomagnetic beads.

Toward the clinical trial, it is significant to evaluate new technologies based on technical specifications. For example, capture efficiency is the most critical parameter in CTCs' isolation. It describes how many CTCs are captured by the microfluidic chip with respect to spiked CTCs. Precisely enumerating almost every single CTC is significant due to the rarity of CTCs in a limited blood sample. Purity, another critical parameter, is how many CTCs captured among hematological cells not purposely captured. High purity would contribute to preventing leukocyte buildup on the surfaces of the microfluidic chip. Specificity is the ability to recognize CTCs even with some disturbance such as WBCs and RBCs. Specificity has to be high for CTCs to be identified among interference. Specificity has to be acceptable with minimum non-specific adhesion. Viability is whether CTCs are viable after captured. Viability would facilitate the following culture and further treatment. A low CTC damage rate is preferred, which avoids a sharp edge, and high shear rates are recommended. Thus, tumor cells would be kept viable for following biological molecular analysis and reculture. High throughput is to process more blood in unit time. Owing to the short half-life of CTCs, 2–4 h,[23] high throughput is favorable to shorten processing time to keep the viability of CTCs captured. Usually, a high flow rate is required to isolate CTCs faster. Another significant metric in CTCs' isolation, reproducibility, is key for clinical application and usually highly neglected. That is, whether the assay is a success for randomly chosen test statistically. High efficiently detecting CTCs should not be occasional, but it should be successful for each test in the sense of statistics based on responsibility on every patient. The metric of reproducibility should be significantly strengthened for future research.

III. IMMUNOMAGNETIC SEPARATION OF CTCs

Affinity ligand-functionalized micro-/nanoparticles, such as epithelial cell adhesion molecule (EpCAM) antibody-coated magnetic microbeads, are utilized for on-chip CTCs segregation.[98–110] Target cells are coated with microbeads modified with an antibody. They are incubated through mixing and shaking. Through this procedure, CTCs are magnetizing and could be attracted by a magnetic field. During the release, removing the magnetic field, magnetic attraction disappears. CTCs captured could be enriched. Examples of immunomagnetic-based isolation of CTCs are listed as follows.

A. Non-hybrid microfluidic chips

1. An intravascular magnetic wire for retrieving CTCs with high throughput

In 2018, Ophir Vermesh and co-workers proposed an intravascular magnetic wire to retrieve rare biomarkers such as CTCs from the subject's blood *in vivo* at a rich yield.[98] The MagWIRE could be inserted and removed through an intravenous catheter to capture biomarkers. Those biomarkers are prelabeled with injected magnetic particles. In an experiment to evaluate the MagWIRE in a live porcine model, *in vivo* labeling could be achieved, and single-pass capture of viable model CTCs could be performed in less than 10 s. The results are so satisfied with the amount of CTCs 10–80 times the amount in the 5-ml blood draw. Compared with the commercially available Gilupi CellCollector the enrichments are 500–5000 times. For conventional microfluidic chips isolating CTCs, the number of CTCs for 5 ml patient blood is few. Thus, the requirement for capture efficiency is high such as to 90%. However, more number of CTCs enriched brings benefit to re-culture for following biological molecular analysis. This MagWIRE could be performed with such a high throughput to yield more number of CTCs. This design would ease following molecular analysis and therapy. But the disadvantage of this approach is kind of invasive to input this MagWIRE to intravenous catheter to realize capture. Almost the majority of microfluidic chips in isolating CTCs are "non-invasive" and extracorporeal. Non-invasive methods are much safer and easy to operate. Only CTCs coated with magnetic particles could be captured. Therefore, it would harm the viability of CTCs for subsequent culture.

2. A two-dimensional immunomagnetic nano-net

In 2019, Chian-Hui Lai and co-workers proposed a two-dimensional immunomagnetic "nano-net," which was designed and synthesized for efficient isolation of CTCs from whole blood (Fig. 2).[111] The nano-net, namely, Ab@Lipo-MNP-GO, was composed of conjugated antibody molecules on a lipid coated magnetic nanoparticle-graphene oxide sheet complex. Superconducting quantum interference device (SQUID), Fourier-transform infrared spectrometer (FTIR), thermal gravimetric analysis (TGA), dynamic light scattering (DLS), and scanning electron microscope (SEM) were used to characterize the magnetism, chemical composition, and the morphology of the construct. Superior properties prove the high possibility of capture of synthetic "nano-net." Clinical patient samples of oral, colon, and lung cancers were utilized to test the performance. The characteristic feature is a stable, patch-like

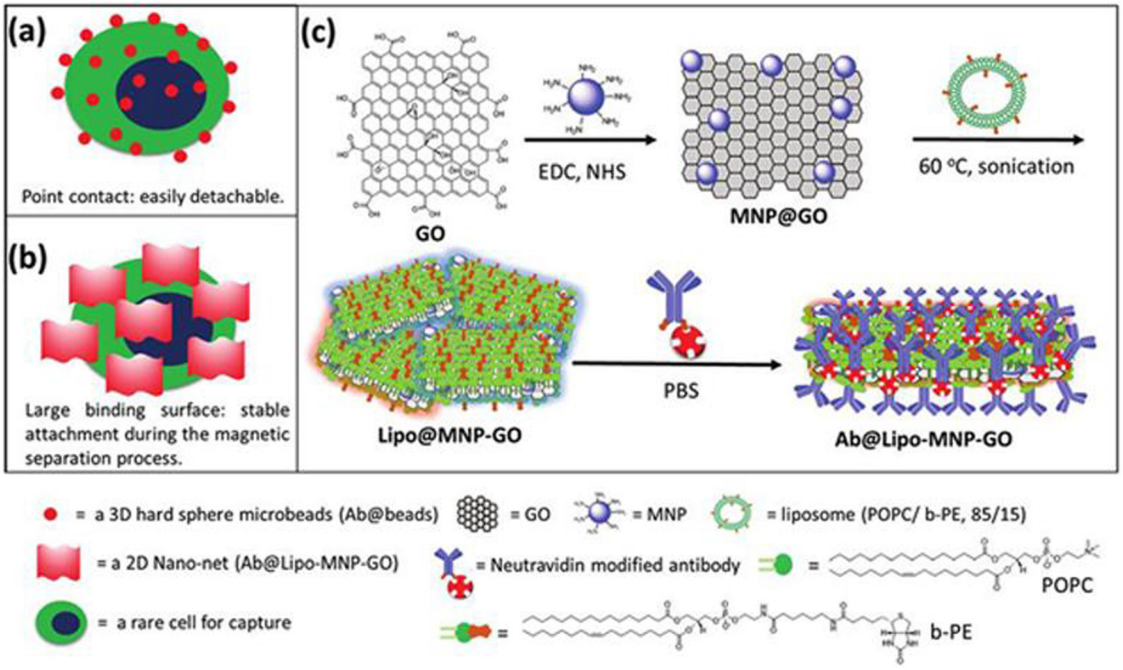

FIG. 2. Schematic illustration of CTCs capture.[111] Reproduced from Lai *et al.*, Nanoscale **11**(44), 21119 (2019). Copyright 2019 American Chemical Society. (a) Immunomagnetic beads, Ab@beads, with point contacts. (b) Patch-like immunomagnetic nano-nets, Ab@Lipo-MNP-GO. (c) Synthetic steps of the nano-net (GO:graphene oxide; MNP@GO: magnetic nanoparticle-GO; Lipo@MNP-GO: lipid bilayer coated MNP-GO; Ab@Lipo-MNP-GO: antibody conjugated lipid bilayer-MNP-GO complex).

multivalent matrix nano-net instead of point contact. From clinical evaluation, the magnetic capture with "nano-net" showed superiority to conventional magnetic capture. Contact areas are greatly enlarged compared to traditional point-to-point contact. Thus, the contact possibility is highly enhanced. However, from the following diagram, it could be seen that the synthetic procedure of this "nano-net" is very complicated. The antibody is very expensive. Comparing with usual "attraction" of magnetic nanoparticles, point-to-point contact has been transferred to area-to-area contact creating more strong chances. Conjugating becomes relatively easy. However, it is difficult for captured CTCs to get rid of this "nano-net." The aim of CTCs capture is following biological molecular analysis. This strong attachment inhibits cell viability and sets up a barricade for subsequent analysis. Therefore, although capture efficiency is highly improved, the "nano-net" is not suitable for clinical application.

3. Microfluidic immunomagnetic detecting chamber

In 2011, Hoshino *et al.* demonstrated the immunomagnetic detection of CTCs.[112] A microchip-based immunomagnetic CTCs' detection described cancer cells labeled with magnetic nanoparticles, entering the chamber, deposited at the bottom of a chamber with arrayed magnets located below (Fig. 3). Customized Fe_3O_4 magnetic nanoparticles conjugated to anti- EpCAM antibodies were added to the blood samples to label cancer cells. In artificial patient blood, capture rates are at 90% and 86% for COLO205 and SKBR3 cells, respectively, even at 10 ml/h. But this magnetic "attraction" would fail into a valid one since the key requirement of every CTC bonded with magnetic beads was not guaranteed. A large number of hematological cells disturb the conjugating of CTCs with magnetic beads. Those CTCs without coating with magnetic nanoparticles escape away, especially at a high flow rate.

4. A combined micromagnetic–microfluidic device

In 2012, Kang *et al.* reported a combined micromagnetic–microfluidic device for rapid capture and culture of rare CTCs (Fig. 4).[113] The device contained a main microfluidic channel and redundant "double collection" channel lined by two rows of dead-end side chambers with a permanent magnet placed directly beneath the lower row of the side chamber to realize tumor cells captured. NdFeB N52 magnet with a magnetic flux density of 14.5 KGs was displaced below the lower row of the collection side chamber of 500 μm. Isolation efficiency was as high as 87% for M6C cells with better than 90% of the isolated CTCs keeping viable after directly tested in the side chambers of the device. Retrieving isolated CTCs from the channels was achieved by shifting the position of the magnet to the opposite side of the device and flushing with PBS. This high-efficient cell separation technique was validated for CTCs circulating in blood of transgenic FVB C3 (1)-SV40T-antigen transgenic mice that experience progression from normal mammary glands to metastatic mammary tumors. As a result, the number of CTCs isolated from the blood of transgenic mice increases with cancer progression. Isolated CTCs from the

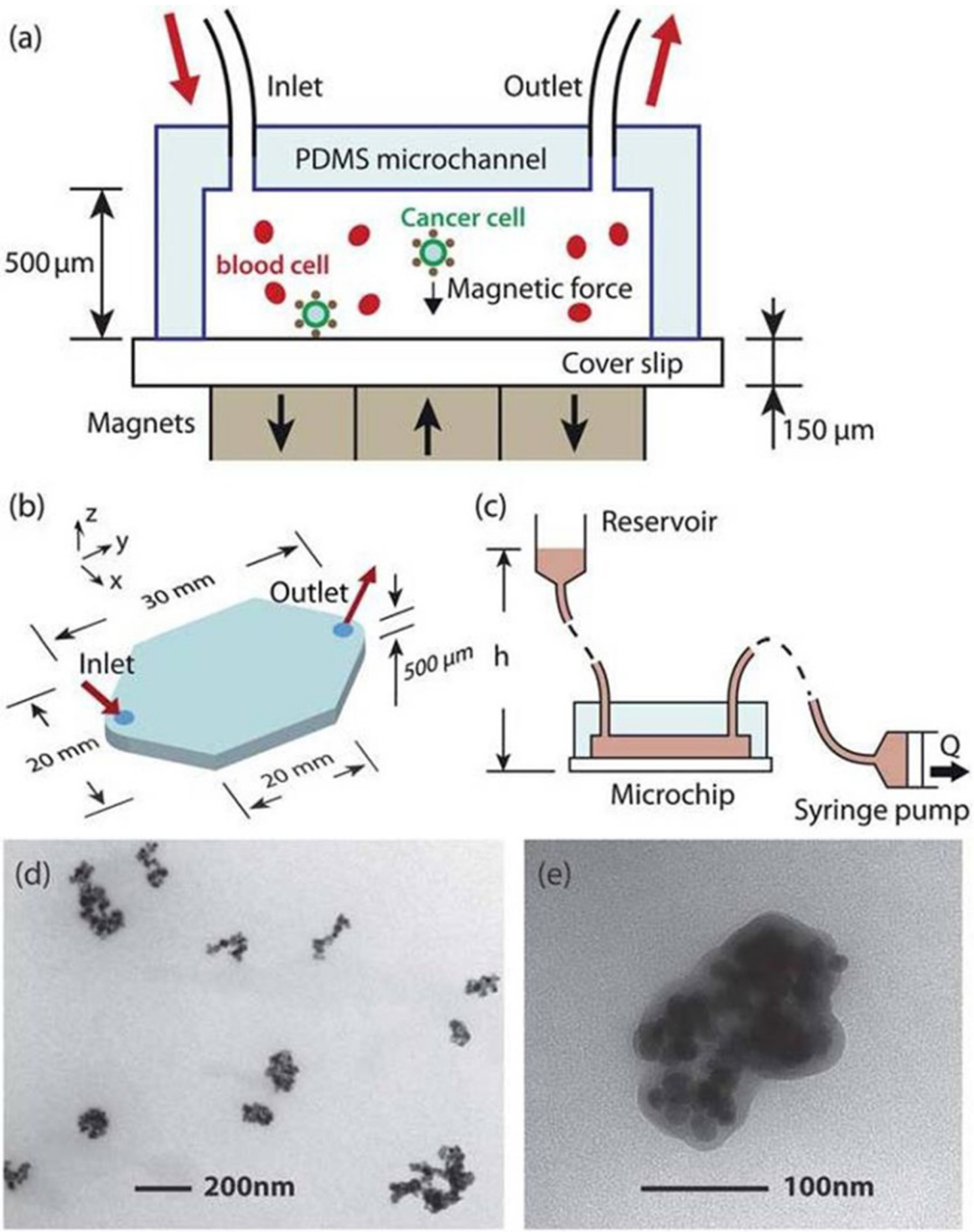

FIG. 3. Microchip design for immunomagnetic detection of the cancer cells.[112] Reproduced with permission from Hoshino *et al.*, Lab Chip **11**, 3499 (2011). Copyright 2011 American Chemical Society. (a) The principle of immunomagnetic capture of CTCs. (b) The dimension of the microchannel. (c) Schematic of the pneumatic flow system. (d) and (e) TEM images of Fe_3O_4 magnetic nanoparticles.

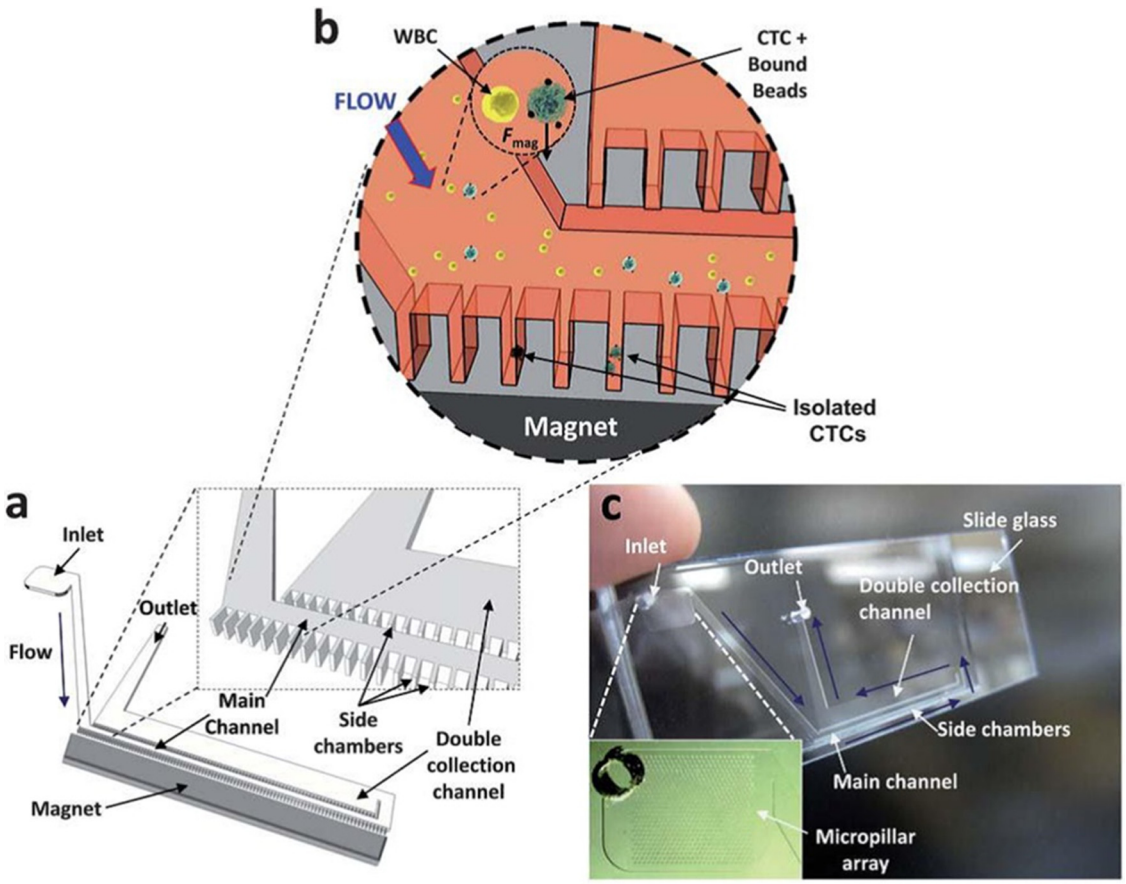

FIG. 4. Design and operation of the immunomagnetic microfluidic chip.[113] Reproduced with permission from Kang *et al.*, Lab Chip **12**, 2175 (2012). Copyright 2012 American Chemical Society. (a) The principle of operation. The device contains an angled inlet channel that connects to the main channel followed by a dual collection channel before going out through the outlet. Rows of dead-end side chambers line the main channel and double collection channel. Permanent magnets are placed beneath the lower row of side chambers to collect magnetic bead-bound cells. (b) Magnified view of part (a). (c) A photograph of a PDMS device.

blood of mice mixing with 4T1 breast tumors proliferated for seven days on a tissue culture plate. In this capture, the side chamber played a significant role in sorting and recovering CTCs, but slim chances still exist for CTCs missing in the main chamber. The reason is that if CTCs could not bond with magnetic nanoparticles, those CTCs would be lost.

5. Self-assembled magnetic bead patterns

In 2010, Sivagnanam demonstrated self-assembled magnetic bead patterns in a microfluidic chip to isolate and on-chip culture of CTCs.[114] Arrays of positively charged (3-aminopropyl)triethoxysilane (APTES) microdots were patterned on a glass substrate and then negatively charged streptavidin-coated magnetic beads were self-assembled on the APTES dots by the external magnetic field. The attached microbeads were modified with biotinylated 5D10 monoclonal antibodies (mAb) and biotinylated fibronectin (FN) in order to capture and culture MCF-7. A NdFeB was placed underneath the microfluidic chip producing a magnetic field of ~100 mT on top of the glass substrate. Capture efficiency could reach 85 ± 10% for cultured MCF-7 cells to be selectively captured from Jurkat cells.

6. Self-assembled magnetic arrays, "Ephesia"

Saliba and co-workers demonstrated an "Ephesia," the self-assembly of super-paramagnetic beads into an array of vertical bead columns (Fig. 5).[115] "Ephesia," formed by dipole–dipole interaction on the magnetic dots from microcontact printing of a ferrofluid onto the glass substrate, trap tumor cells among mixture at a capture yield better than 94%. Magnetic beads with a diameter of 4.5 μm coated anti-CD19 mAb could be self-assembled to form bead columns. Cultivating *in situ* the captured cells were also possible [Fig. 4(b)]. Clinically, this chip is validated with different types of lymphomas and leukemia: chronic lymphocytic leukemia (CLL) (n = number of patients = 4), mantle cell lymphoma (n = 1), and follicular lymphoma (n = 2), and two healthy volunteers. Magnetic

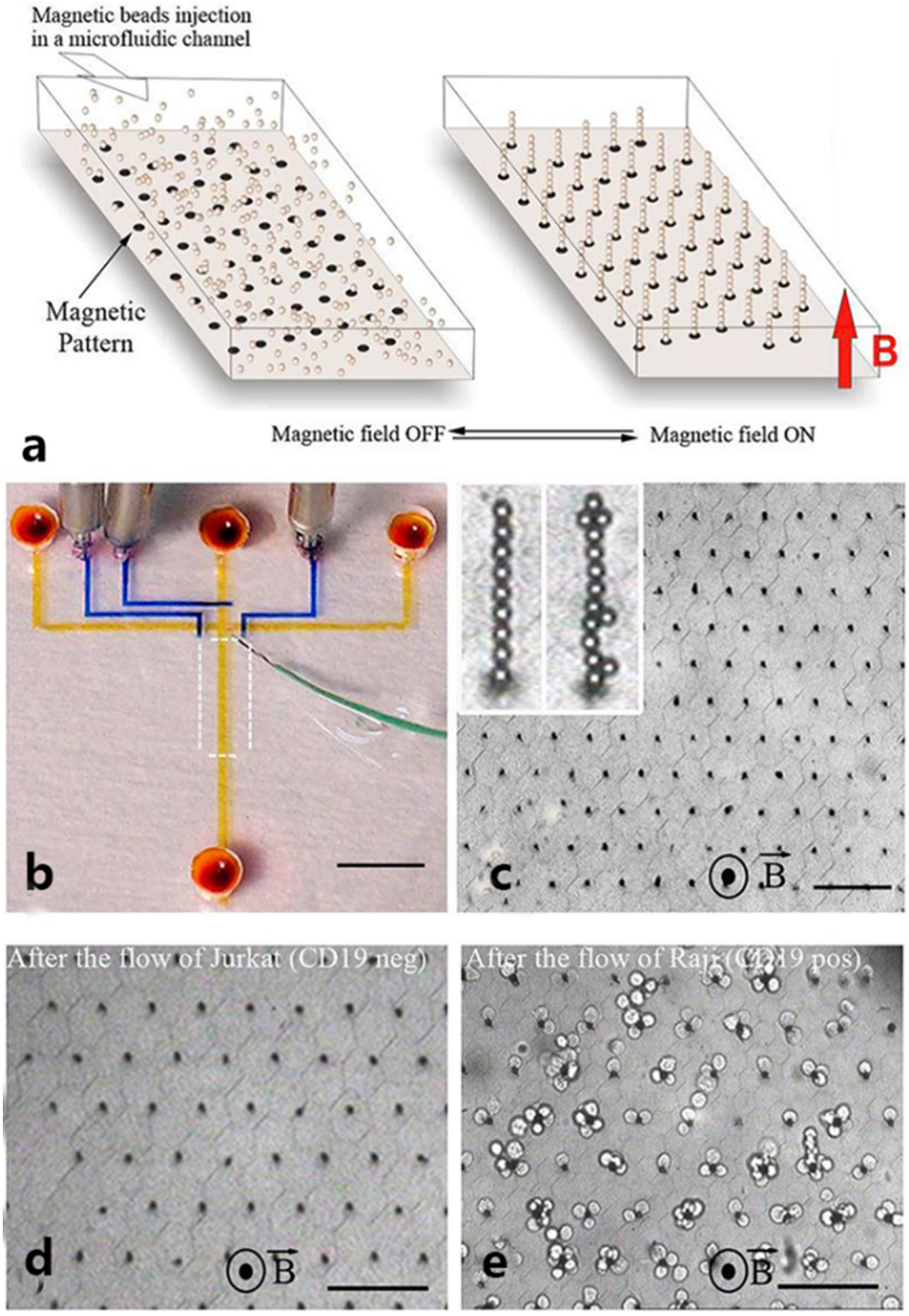

FIG. 5. Principle and practical implementation of Ephesia system.[115] Reproduced with permission from Saliba *et al.*, Proc. Natl. Acad. Sci. U.S.A. **107**, 14524 (2010). Copyright 2010 American Chemical Society. (A) Principle of magnetic self-assembly. Without a magnetic field, beads coated with an antibody are randomly distributed. They would form a regular array of bead columns on top of the ink dots under an external vertical magnetic field. (B) Two levels PDMS integrated microchip. (C) A magnetically assembled array of columns of 4.5 μm beads coated with anti-CD19 mAb. (D) Optical micrograph of the columns after the passage of 1000 Jurkat cells. (E) Captured tumor cells around the columns after the passage of 400 Raji cells.

bead patterns and "Ephesia" are formed in such a fantastic way, automatically producing microposts on the substrate to realize capturing, isolating, and even culture. However, those "artificial" microposts are not strong enough to sustain capture comparing with usual ones, especially when the flow rates have been increased.

7. A surface-enhanced Raman scattering (SERS)-coding microsphere suspension chip

Except for those magnetic attractions, magnetic nanoparticles are gradually and extensively applied to combine with light characteristics. Li *et al.* also creatively figured out a surface-enhanced Raman scattering (SERS)-coding microsphere suspension chip.[116] Folate modified the surfaces of magnetic composite microspheres to capture Hela cells achieving capturing efficiency of 95%. The magnetic supraparticles (MSPs) are composed of high magnetic-responsive supraparticles as a core and a hydrophilic polymeric gel containing targeting molecules as shell. The modified folate (~200 nm in diameter) can be attached to the polymethacrylic acid (PMAA) shell to form MSPs@PMMA-SS-FA. 90% cells could be recovered within 20 min with glutathione solution.

8. A multifunctional biocompatible graphene oxide quantum dots (GOQDs) coated, magnetic nanoplatform

In 2015, Shi and colleagues presented a multifunctional magnetic nanoplatform to selectively separate HepG2 live cancer tumor CTCs from Glypican-3(GPC3)-expression[117] using graphene oxide quantum dots (GOQDs) coated technology. Captured HepG2 tumor cells could be identified on the anti-GPC3-antibody-attached GOQDs-coated magnetic nanoplatform through a two-photon luminescence platform with 960 nm light. The GPC3-specific monoclonal anti-GPC3 antibody, specific to HCC tumor cells, was utilized to attach to the amine-functionalized PEG-coated GOODs-attached magnetic nanoplatforms. Capture efficiency is as high as 97%, and 98% of cells were kept viable after 24 h.

9. Highly qualitatively and quantifiably capture CTCs combining quantum dots (QDs) and magnetic beads

Innovatively, Min *et al.* reported highly qualitatively and quantifiably captured CTCs combining Quantum Dots (QDs) and Magnetic Beads.[118] Anti-EpCAM antibody-conjugated QDs (anti-EpCAM-QDs) target the EpCAM antigen on the surface of the cancer cell for seizing cells. Anti-mouse IgG-modified-magnetic beads (anti-IgG-MBs) function as isolating CTCs with the aid of a magnetic field. Specifically, anti-IgG-MBs could bind to anti-EpCAM antibodies on QDs (anti-EpCAM-QDs). Construction of the topographical objects of QDs could enhance the roughness of CTCs surfaces. This increase enables the multivalent binding of anti-IgG-MBs. As a result, the capture efficiency is 80% for the cells ranging from 100 to 20 cells ml^{-1}, and the capture efficiency for SK-Br3 achieved 70% for 3000 cells mixed with HL-60 cells of 1.0×10^5 in 1 ml of serum-free medium, respectively, with a purity of 18% to 23%. It not only realizes magnetic-based capturing but also technically and creatively introduces QDs to change the morphology of CTCs to serve better sorting. Simple quantification of captured CTCs could be achieved based on the fluorescence intensity of the quantum dots. They use a model cell line, SK-Br3, with a number distributed from 4.0×10^2 to 5.0×10^4. Initially following this conjugation, the fluorescence intensity from the QD-attached cells was measured. A linear relationship was obtained for fluorescence intensity with the number of cells. With this line, the unknown number of cells could be estimated by measuring fluorescence intensity. The detection limit was 390 cells ml^{-1}. This detection and characterization approach is new, and the capture efficiency is increased through enhancing binding with magnetic beads. However, for the clinical patient sample, there are only 1–100 CTCs in 7.5 ml patient blood. Those tumor cells used are beyond the actual number of CTCs, and the capture efficiency is not high enough to perform clinical tests.

10. CTCs' isolation and detection based on ZnS:Mn^{2+} quantum dots and magnetic nanocomposites

Cui presented rapid and efficient isolation and detection of circulating tumor cells based on ZnS:Mn^{2+} quantum dots and magnetic nanocomposites (Fig. 6).[119] Instead of conventional three-color immunofluorescence identification, simultaneously captured CTCs could be achievable due to immunonanocomposites [ZnS:Mn^{2+} quantum dots (QDs) and Fe_3O_4/SiO_2] equipped with permanent fluorescent and magnetic properties. A multifunctional nanocomposite was synthesized by encapsulating ZnS: Mn^{2+} quantum dots (QDs) and Fe_3O_4 nanoparticles into SiO_2 nanospheres and bio-conjugating tumor-specific anti-EpCAM antibodies onto the surface. The synthesized nanocomposite had a high tumor cell binding ability. Fe_3O_4 nanoparticles had a rapid magnetic response that enabled capture of CTCs from patients' blood within minutes. Moreover, the yellow-orange light emitted by the ZnS: Mn^{2+} quantum dots would recognize the cell-immunonanocomposites complexes. CTCs were labeled without utilizing the complicated and destructive procedure utilized in traditional CTCs identification. Up to 90.8% capture efficiency was successfully achieved. Clinically, the specific fluorescence labeling of CTCs was accomplished in nine clinical breast cancer patients' samples.

B. Hybrid microfluidic chips

1. Tumor antigen-independent microfluidic CTC-iChip

Karabacak *et al.* presented a CTC-iChip for marker-free isolation of CTCs from blood samples.[120] CTC-iChip is composed of two separate microfluidic devices.[120] CTC-iChip1 designed an array of posts utilizing continuous deterministic lateral displacement (DLD) to remove nucleated cells and size-based separation of WBCs and tumor cells from whole blood. CTC-iChip2 used inertial focusing to precisely position these cells in a micro-channel and then immunomagnetic isolated CTCs from "magnetic" WBCs coated with magnetic beads. This system could sort up to 10^7 cells/s. Through using two-stage magnetophoresis and depletion antibodies against leukocytes, performance could be achieved of 3.8-log depletion of white blood cells and a 97% yield of rare cells with a sample processing rate of 8 ml of whole blood/h. From the design, we could see that this CTC-iChip structure is a little complex. For DLD separation to isolate CTCs and WBCs for whole blood, CTCs could be lost and there is still RBCs contamination since the number of RBCs is

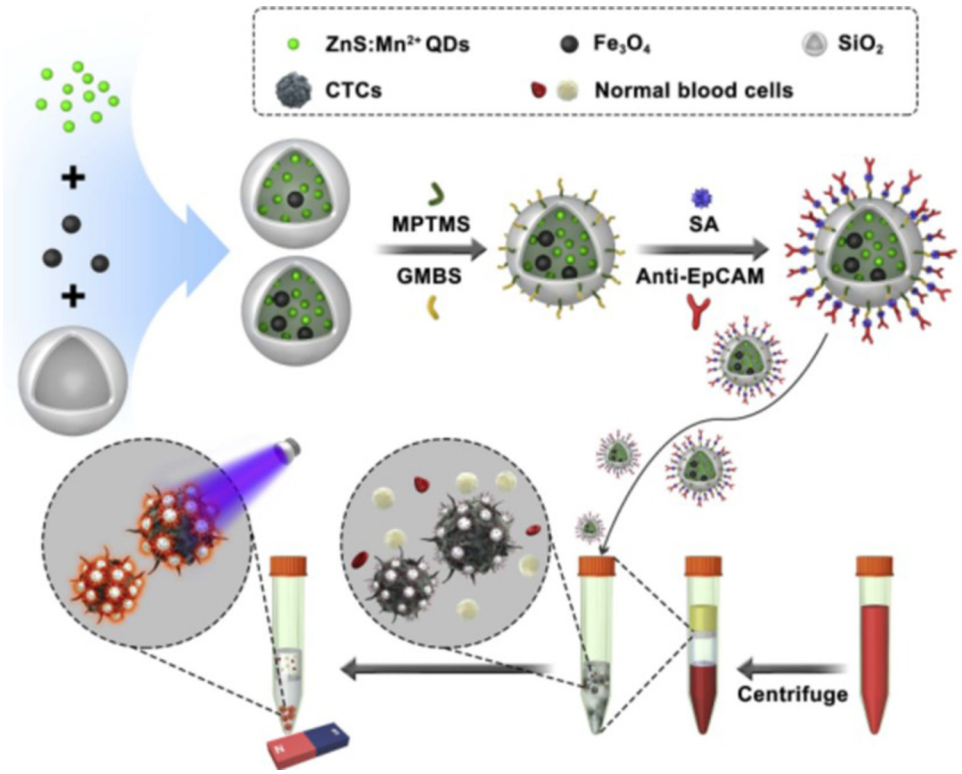

FIG. 6. Scheme showing modifications of the composite material and the procedures for capturing circulating tumor cells.[119] Reproduced with permission from Cui *et al.*, Talanta **202**, 230 (2019). Copyright 2019 American Chemical Society.

enormous. For negative magnetic isolation, the conjugation of WBCs and magnetic beads is not complete although both CD45 and CD66b are used as leukocyte markers.

2. A hybrid magnetic/size-sorting (HMSS) chip for negative enrichment and size-sorting CTCs

This technology is a combination of negative magnetic depletion of leucocytes and the size-selective capture of CTCs (Fig. 7). Chung *et al.* proposed a hybrid magnetic/size-sorting chip. The device named HMSS chip is composed of two parts, a self-assembled magnet filter and a cell-size sorter.[121] The magnetic filter is formed from a layer of magnetic grains (125 μm, NdFeB) self-assembling into an array of anti-aligned magnetic dipoles. A strong magnetic field is produced on the array surface. On top of the device, there is a herringbone structure. The size-based cell sorter is composed of weir-style physical barrier structure with an underpass-gap allowing the passing of small particles such as RBCs. The width of the capture sites is 5 μm. CTCs are trapped at the specific capture sites rendering *in situ* analysis of single cells. Magnetic nanoparticles were attached to leucocytes and mixed at 220 μl/ml cells for 10 min. The flow rate is 3 ml/h. The average recovery rate was 87% for 50 cells and increased to 96% for 250 cells. The technology is a negative depletion of WBCs using incubating WBCs with magnetic nanoparticles. Then, they utilized magnetic "attraction" to get rid of leucocytes and a following size-based sorter to capture tumor cells. Red blood cell lysis (RBL) was used to preprocess the blood. If clinical patient blood was used, the processing procedure is still complicated. CTCs have possibilities to be missed during this procedure, loss in red blood cell lysis (RBL), mixed with WBCs, and remained in the first part of the chip, and pass through the 5 μm gap.

3. A two-stage microfluidic chip for negative enrichment

In 2014, Hyun *et al.* presented a two-stage microfluidic chip for negative enrichment, eluting non-target cells initially (Fig. 8).[122] The first stage included a microfluidic magnetic-activated cell sorting (μ-MACS) chip to eliminate WBCs. Incubate WBCs with magnetic nanoparticles and let the cell suspension pass through a strong magnetic field to remove magnetic WBCs. Then, CTCs enter the second stage to be segregated. The second stage adopted a geometrically activated surface interaction (GASI) chip for selectively isolating CTCs. Modify the microfluidic chip with antibody, anti-EpCAM. CTCs with antigen, EpCAM positive CTCs would bind with antibody modified on the microchannel and be captured. EpCAM negative CTCs without bonding could be collected from the outlet. Deleting WBCs after RBL, then using sorting is a wise technique maximum reducing contamination. However, preprocessing seemed complex such as coating WBCs with magnetic nanoparticles. If not accomplished completely, it would still cause low isolating purity. Utilizing magnets to get rid of WBCs is challenging to perform thoroughly. For the second stage, relying on anti-EpCAM to isolate EpCAM positive CTCs, the capture

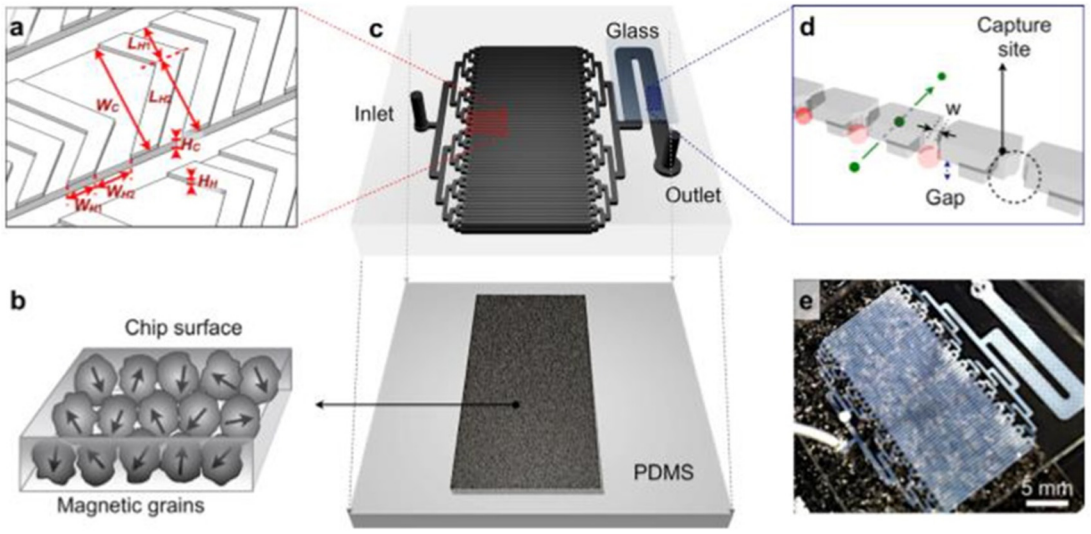

FIG. 7. Design of a hybrid magnetic/size-sorting chip.[121] Reproduced with permission from Chung *et al.*, Biomicrofluidics **7**, 054107 (2013). Copyright 2013 AIP Publishing LLC. (a) A herringbone pattern was integrated on main parallel channels. (b) Self-assembled magnetic grains to generate a strong magnetic field. (c) HMSS sorter. (d) Size-based capture structure. (e) A micrograph of a HMSS sorter.

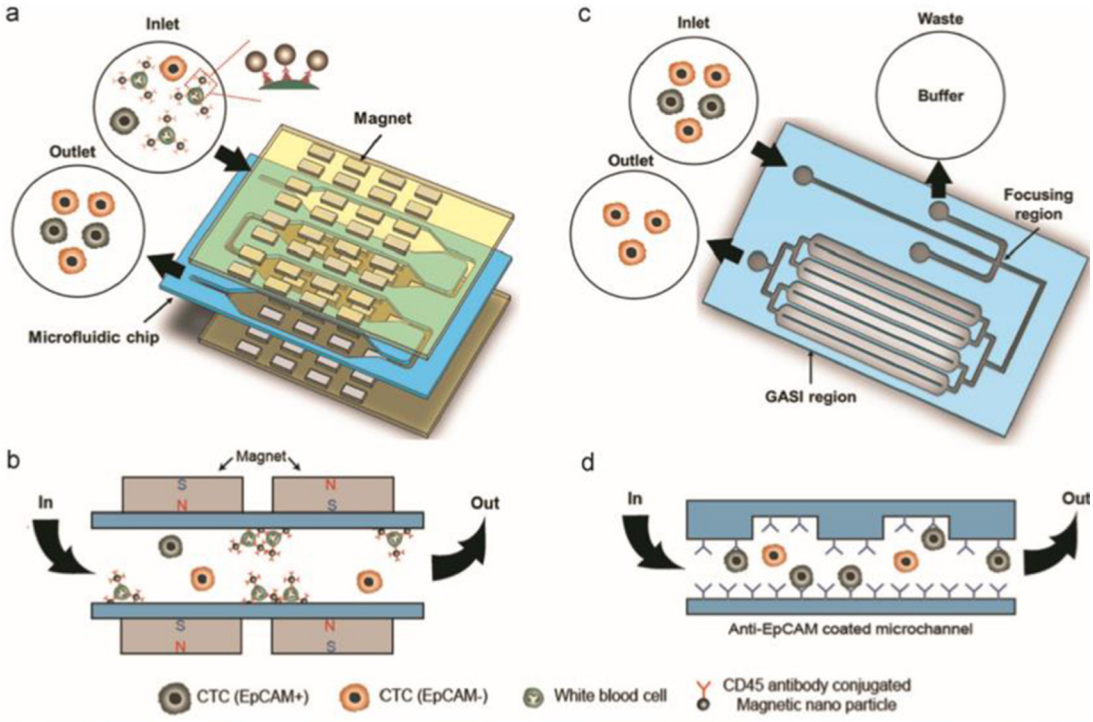

FIG. 8. μ-MACS + GASI.[122] Reproduced with permission from Hyun *et al.*, Biosens. Bioelectron. **67**, 86 (2015). Copyright 2015 American Chemical Society. (a) A two-stage enrichment chip for selective isolation of CTCs. μ-MACS + GASI. (b) μ-MACS was used for negative enrichment to get rid of WBCs. (c) GASI was used for positive enrichment to selectively separate cells based on their surface protein expression. (d) In GASI, anti-EpCAM was coated to microchannel to capture EpCAM positive tumor cells and EpCAM negative tumor cells were flowed away and collected.

efficiency is not high enough to distinguish two kinds of CTCs still with WBCs disturbance.

4. An integrated ferrohydrodynamic cell separation (iFCS) system

Zhao *et al.* proposed an iFCS system (Fig. 9).[123] The enrichment process is composed of two stages integrated on a single iFCS device. The procedure is cell-size variation inclusive and tumor antigen-independent enrichment of viable CTCs. At the same time, WBCs could be depleted. WBCs are bonded with magnetic microbeads through leukocyte surface biomarkers. Therefore, the overall magnetization of the WBC-bead conjugates was more significant than surrounding ferrofluids. The magnetization of the unlabeled CTCs was less than ferrofluids. In the first stage, a magnetic field gradient was generated. Unlabeled and sheath-focused CTCs were pushed to maintain at the upper boundary of a microchannel. However, unbound magnetic beads and WBCs conjugated with ≥3 microbeads were attracted to be depleted toward a waste outlet. In this stage, a high percentage of WBCs and beads are removed to mitigate bead aggregation. In the second stage, a symmetric magnetic field was produced with the highest value in the middle. The "magnetic" WBCs were still attracted at the center of the channel. Unlabeled CTCs were moving along upper and lower boundaries to be collected. Each magnet had a remnant magnetization of 1.48 T. The average recovery rate across eight cancer cell lines was 99.08%. Cell viability of HCC 1806 BrC cells before and after enrichment was determined to be 98.30 ± 0.56% and 97.69 ± .56% for low CTC occurrence rate (1–10 cells ml^{-1}). A blood processing throughput is 12 ml/h. For clinical tests, sizes of CTCs and WBCs were measured for the effective diameter (maximum ferret diameter of cells from their bright-field images) for three breast cancer patients' samples. Analysis of surface antigens expression of individual CTCs was carried out to reveal a high heterogeneity of epithelial and mesenchymal characteristics in these cells. All the design, basic ideas, and experimental results are excellent. However, the whole procedure is relatively complex. RBL is the first one. Magnetic depletion for two stages depends on whether WBCs could conjugate with

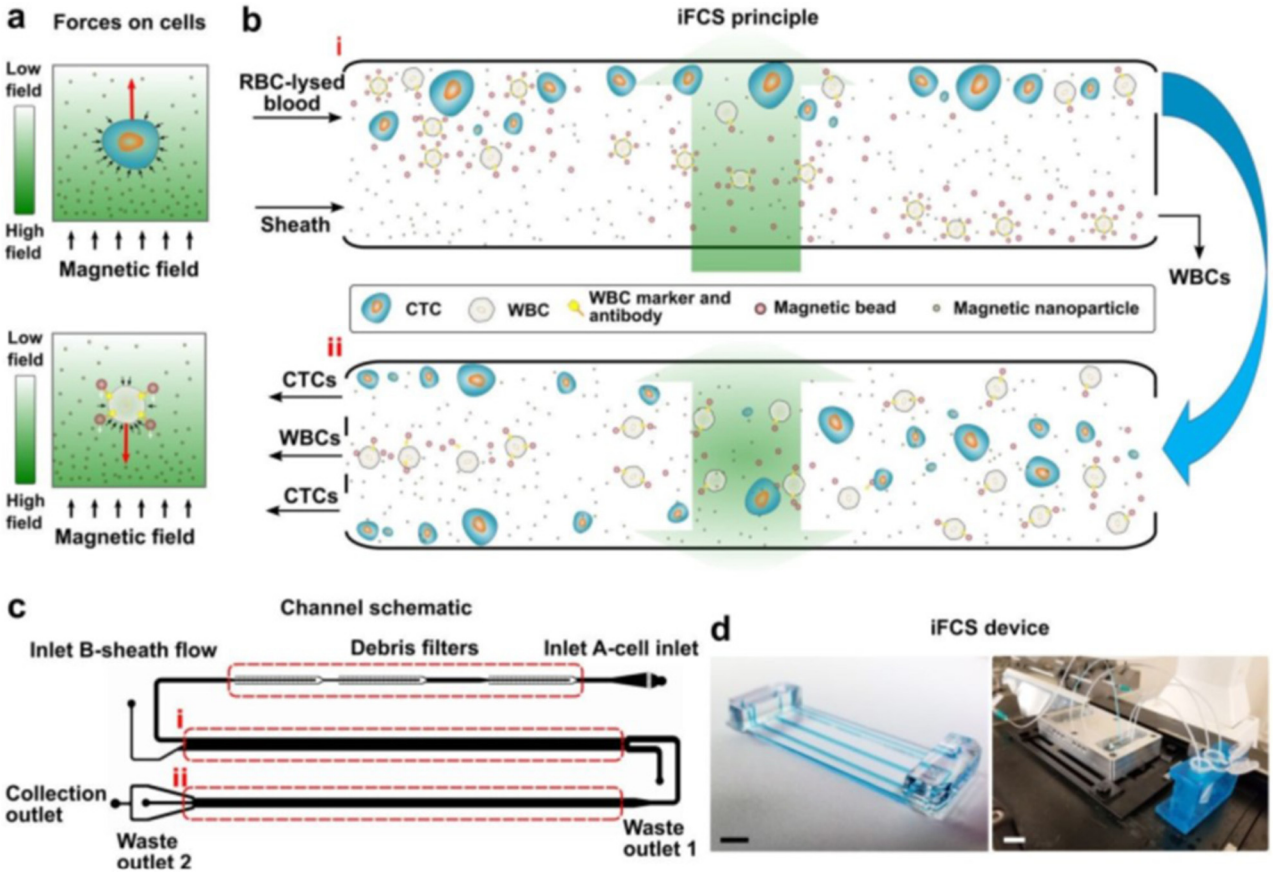

FIG. 9. Overview of an integrated ferrohydrodynamic cell separation (iFCS) system and its working principle.[123] Reproduced with permission from Zhao *et al.*, Lab Chip **19**, 1860 (2019). Copyright 2019 American Chemical Society. (a) Top: schematic of an unlabeled CTC and its moving. Bottom: schematic of a magnetic bead labeled white blood cell (WBC) and its movement. (b) Two enrichment stages for CTCs and magnetic depletion of WBCs. (c) Top-view of the iFCS microchannel. (d) A photo of prototype microchannel (left) and assembled iFCS device with four permanent magnets in quadrupole configuration inside a holder (right).

TABLE I. Summary of magnetic-based method for CTCs isolation (MNP, magnetic nanoparticles, CE, capture efficiency).

Structure	Antibody/Aptamer	Cell line (sample)	CE	Viability	Throughput	Reference
An intravascular magnetic wire[97]	Biotinylated anti-human CD326 (anti-EpCAM) antibody Clone 9C4	H1650 NSCLC cells, Human PCS-201 fibroblasts cells	49%	…	5 cm^{-1}	97
DNA mimic of double-sided tape (DST)[102]	A long single-stranded DNA containing multiple copies of aptamers	T47D, MDA-MB-231	85%	…	…	102
Microfluidic chip (PDMS), NPs[112]	Anti-EpCAM	COLO-205 in blood	90%		10 ml/h	112
Self-assembled magnetic bead patterns in a microfluidic chip[114]	5D10 mAb or biotinylated FN	MCF-7, human leukemia cell line Jurkat	$85 \pm 10\%$	…	0.18–0.36 ml/h	114
Ephesia[115]	Anti-CD19 mAb	Jurkat cell line and Raji cell line	$97 \pm 1\%$	…	$100 \pm 10\,\mu$m/s	115
ZnS:Mn2+ quantum dots and magnetic nanocomposites[119]	Anti-EpCAM	SW480, MCF-7	90.8	…	…	119
μ-MACS + GASI, CD45 antibody-conjugated MNP[122]	CD45 depletion cocktail, Anti-EpCAM	MCF-7,MDA-MB-231, SK-BR-3	98.81%	…	2.4, 6 ml/h	122
A semi-integrated electrical biosensor[123]	Anti-EpCAM	MCF-7 in blood	>70%	…	1.2 ml/h	123
A combined microfluidic micromagnetic device[124]	Anti-EpCAM	M6C, 4T1 in blood	90%	>90%	1.2 ml/h	113
Immunomagnetic gold hybrid nanoparticles[124]	Anti-EpCAM	MCF-7, leukocytes, Jurkat T cells	79%	…	…	124
A chip-based DMR system, Mn-MNP[125]	Anti-HER2/neu, anti-EGFR, anti-EpCAM	HCT116, SkBr3, MDA-MB-231 in leukocytes	…	…	…	125
Miniaturized diagnostic magnetic resonance (DMR), MNP[126]	Her2/neu, EGFR	3T3, SK-BR-3, MDA-MB-231	…	…	…	126
A micro-nuclear magnetic resonance system[127]	EpCAM,MUC-1, Her2, EGFR, B7-H3, CK18, Ki-67, p53	50 patients	…	…	…	127
An electrical biosensor[128]	Anti-EpCAM	MCF-7	>88%	…	9 ml/h	128
Magnetic sifters, MNP[129]	Anti-EpCAM	H-1650 (NSCLC)	95.7%	…	10 ml/h	129
A magnetic capture column and a size-selective filter[130]		GCIY-EGFP, RPMI1788	97.7%, 97.5%	97.6%	30–1200 ml/h	130
Magnetic capture (Apt-MBs)[131]	Aptamer conjugated magnetic beads	DLD-1, CCRF-CEM	73%, 55%	…	…	131

magnetic beads. Since the amount of WBCs is massive all WBCs would not definitely bond with magnetic nanoparticles.

The immunomagnetic separation of CTCs of all kinds of microfluidic chips has been summarized in Table I.

The summary of commercially available systems is listed in Table II.

IV. DISCUSSION

The immunomagnetic separation described above adopted a complicated approach of conjugating magnetic nanoparticles. Some of them are through bonding WBCs with magnetic beads and then utilizing magnetic "attraction" to get rid of WBCs. Modifying the microchannel with antibodies such as anti-EpCAM to capture EpCAM expressed CTCs and separately collected mesenchymal-expressed CTCs. Then only mesenchymal-expressed CTCs are left. Therefore, we collect them to achieve separation of two kinds of CTCs. Positively, magnetic beads connected with CTCs. In order to increase contact chances, CTCs conjugate with QD first to increase roughness and then connect with magnetic beads. In order to increase the contact area, traditional point-to-point contact has been transferred to "nanonet." From the description above, it could be seen complex processing procedure has been taken. QD, GOQD, ZnS:Mn^{2+} quantum dots, SERS mark, and magnetic beads modifying with kinds of antibodies are to increase affinity contact. Our aim to perform rich CTCs experiments is to carry out clinical assays and

TABLE II. Summary of commercially available systems.

CTC commercial system	Principle	Reference
CellSearch	The CellSearch system (Veridex, Raritan, NJ, USA) is the only US FDA-approved CTC enumeration system.[132] Utilizing ferrofluid nanoparticles with antibodies that target epithelial cell adhesion, CTCs are magnetically separated from the bulk of other cells in the blood. This system could successfully enumerate breast, prostate, and colorectal cancers through immunomagnetic technology. Disadvantages are high cost and low efficiency.	132
IsoFlux	The IsoFlux System (Fluxion Biosciences Inc., South San Francisco, CA) could provide high-sensitivity rare cell isolation coupled with a novel cell retrieval mechanism.[133] The system employs immunomagnetic beads that facilitate use of single or multiple capture antibodies to target cells of a specific pathology. With an externally applied magnetic field the sample flow over a microfluidic device that contains an isolation zone to capture CTCs on the upper surface of the cartridge. The roof of the microfluidic channel could detach from the rest of the cartridge and transfers off-chip with the CTCs remained on its surface, providing near perfect transfer efficiency. It is possible for the IsoFlex System to molecular characterize intact viable CTCs or isolated DNA, RNA, or protein.	133
ImageStream	The ImageStream (Amnis, Seattle, WA, USA) is an imaging cytometry device that combines the strengths of flow cytometry and fluorescent microscopy in a single platform and has potential in applications to CTC counting.[134] ImageStream platform for CTC enumeration potentially has a value for the early diagnosis of disseminated disease. Disadvantage is precision needed for the enumeration of low number of CTC.	134
Clearbridge BioMedics' ClearCell FX System	The ClearCell FX1 System works as a leading, automated, label-free liquid biopsy device for the enrichment of target cells from blood.[79] The system employs the innovative microfluidic CTChip to isolate and retrieve intact, viable target cells from a patient's blood sample. This is a spiral microfluidic chip that could size-based separate and retrieve CTCs from blood with high throughput using the Dean drag force coupled with inertial microfluidics phenomenon.	79
Celsee Diagnostics CTC system	Key component of this system is a microfluidic chip with individual compartment in the shape of a bowl as the cross section and with a 7.5 μm width tunnel located below as filter.[135] Blood sample was injected from top and enriched in the bowl and hematological cells and waste flow away from the bottom. It is based on size and deformability. The Celsee PREP 400™ system (Celsee Diagnostics, Plymouth, MI) is an automated system of this microfluidic device and contains a parallel network of four microfluidic chips each of which has approximately 56 320 capture chambers. The downstream of identification of individual CTCs can use a wide array of antibodies and DNA/RNA-based probes.	135
Parsortix cell separation system from ANGLE plc Company	Parsortix™ Cell Separation System (ANGLE North America, Inc., King of Prussia, PA) is a microfluidic based technology capturing CTCs based on size and deformability.[136] It has reproducibly high capture efficiency and obtains highly enriched, viable (viability dependent on preservative used) CTCs that are amenable to a multitude of downstream analysis even for single cells. There exists a Parsortix GEN3 Cell Separation Cassette showing how the blood flows into the cassette, over the step structures and through the critical gap. The separation cassette is with a 6.5 μm critical gap size.	136

come out following treatment. High-cost antibody and complex processing processes hinder clinical tests, especially without reproducibility. Due to the lack of clinical trials to validate the microfluidic chips, the significance of the design and experiments is questionable. Clinical validation requires the success of each patient blood test, which puts a high requirement of the reproducibility of microfluidic chips. However, reproducibility is almost neglected in those assays and most CTC chips. With advanced progress of CTC chips, other easy-operate and fast approach has been introduced into this area such as laser detection. Utilizing laser irradiating on the blood samples or processed ones, the characteristic spectrum would tell everything. The location of characteristic peaks can be used to identify cancer type, and the intensity can be determined by the number of CTCs. Magnetic separation combining with light technology would bring "revolutionary" CTCs' isolation. Utilizing "magnetic beads," we would further purify the blood sample to go a step further to serve light

identification better. Laser identifying needs more number of CTCs with satisfactory purity. With magnetic attraction and other technology to get rid of most hematological cells such as RBCs and WBCs, light identifying that even could be accomplished within several seconds could be realized and come earlier.

V. CONCLUSIONS

Immunomagnetic separation of CTCs is very sensitive, specific, and not limited to the flow rate with no need for modification of surfaces of the microchannel. The release is simple to be realized by removing the magnet. The only FDA-approved product of CellSearch is based on this approach. Nevertheless, target cells have to bind with affinity ligand-functionalized micro-nanoparticles. The bonding efficiency determines the capture effectiveness. A large number of hematological cells would interfere with CTCs bonded with immunomagnetic particles, causing ineffectiveness. To promote CTC-beads' binding, red blood cell lysis is usually taken. Cell viability is also affected to some extent. Same as the affinity-based method, it would depend on the expression of certain surface markers. Therefore, it would be better to adopt a size and magnetic combination.

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DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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